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(57) Abstract

The present invention relates generally to improved methods of using 4–quinoline–carboxylic acid derivatives, particularly brequinar, for the treatment of various medical conditions, including cancer, arthritis, inflammatory disorders, and organ transplantation rejection. These methods improve the therapeutic effectiveness and reduce the toxicity of the 4–quinoline–carboxylic acid derivatives.

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IMPROVED THERAPEUTIC USES OF 4-QUINOLINE CARBOXYLIC ACID DERIVATIVES

BACKGROUND OF THE INVENTION

The present invention relates generally to improved methods of using 4-quinoline-carboxylic acid derivatives, particularly brequinar, for the treatment of various medical conditions, including cancer, inflammatory disorders, and organ transplantation rejection.

Cinchophen, 2-phenyl-4-quinoline carboxylic acid, has been known for many years and has been described as being useful as an antirheumatic and in the treatment of gout. However, it can produce a possibly precancerous degeneration of liver tissue. Other 4-quinoline carboxylic acid derivatives have been reported to have anti-neoplastic, anti-inflammatory and immunosuppressive effects, and to be useful for treating cancer, inflammatory disorders, and organ transplant rejection. For example, U.S. Patent No. 4,680,299 (Hesson) reports the use of 2-phenyl-4-quinoline carboxylic acid derivatives for inhibiting the growth of mammalian tumors. U.S. Patent No. 4,847,381 (Sutherland et al.) reports the use of substituted 4-quinoline carboxylic acids in the treatment of arthritis and inhibition of progressive joint disease. U.S. Patent No. 4,968,701 (Ackerman et al.) reports the use of 4-quinoline carboxylic acid derivatives as immunomodulatory and anti-inflammatory agents, for the treatment of, among other conditions, organ transplantation rejection. U.S. Patent No. 5,204,329 (Ackerman et al.) reports methods of use of 4-quinoline carboxylic acid derivatives in combination with other immunosuppressive agents to treat organ transplant rejection and other immune regulatory diseases; according to Ackerman et al., the combination has a synergistic effect and results in fewer and less severe side effects than does use of immunosuppressive agents alone. U.S. Patent No. 4,861,783 (Ackerman et al.) reports the use of substituted quinoline carboxylic acid derivatives in treating skin and muco-epithelial diseases. U.S. Patent No. 4,968,702 (Poletto et al.) addresses substituted quinoline carboxylic acid derivatives, methods for their use in treatment of

arthritis and other joint degenerative diseases, and their use in inducing immunosuppression. U.S. Patent No. 5,428,040 (Magolda et al.) reports the use of fused-ring quinoline carboxylic acid derivatives in the treatment of organ transplantation rejection and cancer, either alone or in combination with (1) one or more immunosuppressive agents, or (2) non-steroidal anti-inflammatory agents, or (3) tumor inhibiting agents, i.e, 5-fluorouracil.

In particular, a derivative known as brequinar sodium (NSC368390;DuP785) [6-fluoro-2-(2'-fluoro-1-1'-biphenyl-4-yl)-3-methyl-4-quinoline carboxylic acid sodium salt] was originally developed as an anti-proliferative agent for the treatment of cancer. [Noe et al., *Cancer Res.*, 50:4595-4599 (1990); Cody et al., *Am. J. Clin. Oncol.*, 16:526-528 (1993); Dexter et al., *Cancer Res.*, 45:5563-5568 (1985).] Brequinar was reported to have activity against L1210 leukemia, P388 leukemia, colon 38 carcinoma and B16 melanoma tumors *in vivo* in mice as well as have activity against several human cancer cell lines (LX-1 lung, HCT-15 colon, MX-1 breast, BL-STX-1 stomach and CX-1 colon) grafted into animals. Brequinar was also reported to inhibit lung metastasis of B16 F10 melanoma in mice and to inhibit growth of classic and variant small cell lung cancer cells and human DLD-1, clone A and DLD-2 colon cancer cells *in vitro*. [Arteaga et al., *Cancer Res.*, 49:4648-4653 (1989); Schwartsmann et al., *Cancer Chemother. Pharmacol.*, 25:345-351 (1990).]

Biochemical studies demonstrated the ability of brequinar to inhibit the enzymatic activity of dihydroorotate dehydrogenase (DHO-DHase), the fourth enzyme of the *de novo* pyrimidine biosynthetic pathway. Inhibition of this enzyme prevents the formation of pyrimidine nucleotides necessary for the synthesis of RNA and DNA, thus blocking cell proliferation. The importance of the pyrimidine synthetic pathway to T cell proliferation has been elegantly demonstrated by Fairbanks et al., *J. Biol. Chem.*, 270:29682-29689 (1995). They demonstrated that the metabolic requirements of pyrimidines in resting T lymphocytes were met by salvage, but upon stimulation to T cells with PHA under culture conditions where exogenous uridine was limiting, the

synthetic pathway was critical for the observed 8-fold expansion of pyrimidine nucleotide pools. These data supported the prevailing widely accepted hypothesis that the immunosuppressive activity of brequinar occurs by inhibition of DHO-DHase which ultimately prevents clonal expansion of activated T cells. The structures and substituents involved in DHO-DHase inhibition have been further studied in Chen et al., *Biochem. Pharmacol.*, 40:709-714 (1990), which identified three critical regions: (1) the C2 position, where bulky hydrophobic substituents are necessary, (2) the C4 carboxylic acid substituent, and (3) the benzo portion of the quinoline ring with appropriate substitutions.

Brequinar has been described to exhibit a number of immunosuppressive activities. *In vitro*, brequinar inhibits T and B cell proliferation, as well as antibody production. *In vivo*, brequinar suppresses the induction of contact sensitivity, the generation of cytotoxic T cells and the production of antibody. Brequinar also prevents the rejection of allografts and xenografts in a number of rodent transplant models. Because clonal expansion of lymphocytes is central to the induction of both humoral and cellular immune responses, it became widely accepted that immunosuppression by brequinar is mediated through inhibition of pyrimidine synthesis. [Makowka et al., *Immunol. Rev.*, 136:51-70 (1993); Makowka et al., *Transplant. Proc.*, 25:2-7 (1993).] However, patients with defects in the *de novo* pyrimidine pathway (in hereditary orotic aciduria) did not appear consistently immunocompromised, although they are consistently anemic.

However, the use of brequinar as an anti-neoplastic, anti-inflammatory or immunosuppressive agent has been limited by its toxic side effects, including bone marrow suppression, which manifests as anemia, leukopenia, granulocytopenia, thrombocytopenia, or bone marrow hypoplasia. [Makowka et al., *Immunol. Rev.*, 136:51-70 (1993); Makowka et al., *Transplant. Proc.*, 25:2-7 (1993).] In fact, clinical trials of brequinar in humans for the treatment of various cancers, including advanced breast cancer, colo-rectal, head and neck, kidney, melanoma, lung, ovarian, prostate, and

other solid tumors, encountered difficulty with dose escalation because of excessive toxicity. The maximally tolerated doses reported in Arteaga et al., *Cancer Res.*, 49:4648-4653 (1989) were 250 mg/m^2 given intravenously daily for 5 days for good risk patients, and 135 mg/m^2 daily for 5 days for poor risk patients (extensive and high dose prior chemotherapy or radiotherapy). In Noe et al., *Cancer Res.*, 50:4595-4599 (1990), the maximum tolerated dose was 300 mg/m^2 daily for 5 days. The maximally tolerated doses reported in Schwartsmann et al., *Cancer Chemother. Pharmacol.*, 25:345-351 (1990) were 2,250 mg/m^2 for good risk patients and 1,500 mg/m^2 for poor risk patients, given by a brief intravenous infusion once every 3 weeks. In Cody et al., *Am. J. Clin. Oncol.*, 16:526-528 (1993), the starting dose of 1800 mg/m^2 given intravenously weekly resulted in unacceptable toxicity, and median weekly doses were reduced to 1200 mg/m^2. The non-hematological dose-limiting toxicities were largely mucocutaneous side effects such as dermatitis, mucositis, stomatitis and skin rash, which could result in sloughing of skin. Other side effects included nausea, vomiting, fatigue, reversible elevation of liver enzymes and diarrhea.

It had been previously reported that topical uridine was able to improve the mucocutaneous side effects of another pyrimidine biosynthesis inhibitor, N-phosphonacetyl-L-aspartate (PALA), and Schwartsmann et al., *Cancer*, 63:243-248 (1989) suggested that topical uridine might be tested in patients developing skin toxicity due to brequinar.

Woo et al., *Transplantation*, 56:874-881 (1993) reported that addition of brequinar to a spleen cell culture resulted in inhibition of proliferation as measured by uptake of radiolabelled thymidine, and that the further addition of exogenous uridine reversed brequinar's inhibitory effect on nucleotide synthesis, while the addition of exogenous cytidine worsened brequinar's inhibitory effect on nucleotide synthesis.

Therefore, there exists a need for improved methods of using 4-quinoline carboxylic acid derivatives to treat medical conditions. Ideally such

improved methods would provide reduced toxicity and would maintain comparable or even increased therapeutic effectiveness.

SUMMARY OF THE INVENTION

The present invention provides improved methods of using 4-quinoline-carboxylic acid derivatives, particularly brequinar, for the prophylactic or therapeutic treatment of various medical conditions, including cancer, arthritis, inflammatory disorders, and organ transplantation rejection. According to the invention, the co-administration of a pyrimidine, such as uridine, with the 4-quinoline-carboxylic acid derivative reduces its toxicity while maintaining its therapeutic effectiveness.

The brequinar may also be administered in conjunction with other known anti-neoplastic, anti-inflammatory or immunosuppressive agents.

A further aspect of the invention involves use of a pyrimidine for the manufacture of a medicament for co-administration with a 4-quinoline-carboxylic acid derivative, and 4-quinoline-carboxylic acid derivative for the manufacture of a medicament for co-administration with a pyrimidine, as well as use of a pyrimidine and a 4-quinoline-carboxylic acid derivative together for the manufacture of a medicament.

Numerous additional aspects and advantages of the invention will become apparent to those skilled in the art upon considering the following detailed description of the invention, which describes the presently preferred embodiments thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A depicts hematocrits at 3, 4 and 6 weeks of mice treated with nothing (control) or brequinar at 10 or 20 mg/kg, reported as % packed cell volume (PCV). Figure 1B depicts hematocrits of mice (% PCV) at six weeks after treatment with various doses of brequinar or a combination of brequinar and uridine.

Figures 2A and 2B depict the average weight of the lymph nodes and spleen, respectively, for mice treated with brequinar, uridine, or a combination of both.

Figure 3 depicts hematocrits of mice (% PCV) at 13, 23, 31 and 53 days after treatment with brequinar, uridine, or a combination of both.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides improved methods of using 4-quinoline-carboxylic acid derivatives for the treatment of various medical conditions, including cancer, arthritis, inflammatory disorders, including auto-immune diseases, and organ transplantation rejection, including graft vs. host disease. According to the invention, the co-administration of a pyrimidine with the 4-quinoline-carboxylic acid derivative reduces its toxicity while maintaining its therapeutic effectiveness. As used herein, a "4-quinoline-carboxylic acid derivative" is defined to include those compounds described as having anti-neoplastic, anti-inflammatory and/or immunosuppressive activity in U.S. Patent Nos. 4,680,299 (*e.g.*, at col. 4, line 30 to col. 8, line 40), 4,847,381 (*e.g.*, at col. 1, lines 1-35), 4,968,701 (*e.g.*, at col. 1, line 20 to col. 3, line 48), 5,204,329 (*e.g.*, at col. 2 to col. 4, line 50), 4,861,783 (*e.g.*, at col. 1, line 48 to col. 4, line 15), 4,968,702 (*e.g.*, col. 1 to col. 2), and 5,428,040 (*e.g.*, col. 7, line 45 through col. 10, line 25), all of which are hereby incorporated by reference, and which also describe methods of preparing such derivatives and therapeutic uses for such derivatives. A preferred 4-quinoline-carboxylic acid derivative is brequinar. As used herein, a "pyrimidine" includes compounds useful either directly or as intermediates in pathways for supplying pyrimidine nucleotides (uridine, cytidine and thymidine). A preferred pyrimidine is uridine. Other suitable pyrimidines include the pyrimidine intermediates orotic acid and orotidine. Other exemplary pyrimidines include cytidine and thymidine, possibly at higher doses.

The invention is based in part on the discovery that the *in vivo* pharmacological activity of 4-quinoline-carboxylic acid derivatives is mediated by their inhibition of tyrosine kinase phosphorylation, which plays an important role in the activation and proliferation of lymphocytes following the stimulation of antigen and cytokine receptors, and that the activity of these drugs is largely independent of their inhibition of the DHO-DHase enzyme in the pyrimidine synthetic pathway. This discovery is directly contrary to the prevailing belief that the pharmacological activity of these compounds is mediated by the inhibition of pyrimidine synthesis. In fact, the inhibition of pyrimidine synthesis results in suppression of bone marrow hematopoiesis and is responsible for the toxicity of these compounds. This toxicity can effectively be ameliorated by administration of a pyrimidine, without impairing the therapeutic effectiveness of the 4-quinoline-carboxylic acid derivatives.

Specifically, the results described herein demonstrate that, in a mouse allograft model of transplant rejection and in a mouse model of auto-immune disease and lymphoproliferative disorder, immunosuppression by a 4-quinoline-carboxylic acid derivative, brequinar, is largely independent of the effects on pyrimidine synthesis, while brequinar is active as an inhibitor of protein tyrosine phosphorylation. The results also show that co-administration of a pyrimidine, uridine, with brequinar effectively eliminates the toxicity associated with the inhibition of pyrimidine synthesis while maintaining brequinar's immunomodulatory activity in the transplant rejection, auto-immune disease and lymphoproliferative disorder models. Similar results are expected for the use of brequinar in the treatment of certain types cancer, in view of previous observations that brequinar does not decrease pyrimidine levels in some tumors, and that some tumors are driven by aberrant tyrosine kinase activity, including breast and ovarian cancers, squamous cell carcinoma, gliomas, chronic myelocytic leukemia and acute lymphocytic leukemia. [Levitzki et al., *Science*, 267:1782-88 (1995).] Tyrosine kinase activity has also been implicated in psoriasis, atherosclerosis and TNF-dependent inflammatory conditions.

The improved methods of the present invention are useful for the treatment of subjects undergoing a variety of organ transplants, including kidney, liver, heart, lung, skin, bone marrow and pancreatic islet cells. These improved methods are also useful for the treatment of neoplastic diseases, including leukemia, breast cancer, lung cancers including small cell lung cancer, colon cancers, stomach cancer, melanoma, other skin cancers, and brain cancers. These improved methods are further useful for the treatment of various inflammatory disorders, including lupus erythematosus, Sjogren's syndrome, myasthenia gravis, vasculitis syndromes, and other auto-immune diseases, rheumatoid arthritis, reactive arthritis, osteoarthritis, ankylosing spondylitis, scleroderma, mixed connective tissue disease, sarcoidosis, multiple sclerosis, Crohn's disease, inflammatory bowel disease, primary biliary cirrhosis, and psoriasis.

The 4-quinoline-carboxylic acid derivative and the pyrimidine may be administered systemically or topically. Systemic routes of administration include oral, intravenous, intramuscular or subcutaneous injection (including into depots for long-term release), intraocular or retrobulbar, intrathecal, intraperitoneal (e.g. by intraperitoneal injection or lavage), intrapulmonary using aerosolized or nebulized drug, or transdermal using, e.g., patches. Topical routes include administration in the form of salves, creams, jellies, ointments, ophthalmic drops or ointments, ear drops, suppositories, or irrigation fluids (for, e.g., irrigation of wounds).

When given orally or parenterally, 4-quinoline-carboxylic acid derivative compositions are generally administered in doses ranging from 1 μg/kg to 500 mg/kg per day, or preferably in doses ranging from 2 mg/kg to 50 mg/kg per day, or in equivalent dosing at longer or shorter intervals. For the treatment of cancer, higher dosages of the 4-quinoline-carboxylic acid derivative over a shorter treatment period may be preferred. Brequinar is water soluble and has been reported to have very good oral bioavailability, reaching blood levels comparable to (greater than 90% of) the levels achievable by intravenous injection. [Arteaga et al., *Cancer Res.*, 49:46-48

(1989); Sherls et al., *Hepatology*, 18:746 (1993).] Brequinar has previously been administered to humans intravenously at doses ranging from 15 mg/m^2 to a maximal tolerated dose of 2,250 mg/m^2 (a 70 kg man has a surface area of approximately 1.73 m^2). Prior studies in mice indicated that mice receiving the optimal intravenous dose on a daily schedule for 9 days had a brequinar blood level of 3 μg/ml. [Noe et al., *supra*.] It is contemplated, however, that co-administration with uridine will improve tolerability of brequinar and therefore raise the maximally tolerated dose by at least 5-fold.

Pyrimidine compositions are generally administered in doses ranging from 1 mg/kg to 5000 mg/kg per day, preferably in doses ranging from 50 mg/kg to 200 mg/kg per day given orally, or in equivalent dosing at longer or shorter intervals. Humans with orotic aciduria are generally given uridine supplementation at doses of 150 mg/kg.

The doses of the 4-quinoline-carboxylic acid derivative or pyrimidine may be increased or decreased, and the duration of treatment may be shortened or lengthened as determined by the treating physician. The frequency of dosing will depend on the pharmacokinetic parameters of the agents and the route of administration. The optimal pharmaceutical formulation will be determined by one skilled in the art depending upon the route of administration and desired dosage. See for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712, the disclosure of which is hereby incorporated by reference. Such formulations may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the administered agents.

Those of ordinary skill in the art will readily optimize effective dosages and concurrent administration regimens as determined by good medical practice and the clinical condition of the individual patient. Regardless of the manner of administration, the specific dose may be calculated according to body weight, body surface area or organ size. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely

made by those of ordinary skill in the art without undue experimentation, especially in light of the dosage information and assays disclosed herein, as well as the pharmacokinetic data observed in the human clinical trials discussed above. Appropriate dosages may be ascertained through use of established assays for determining blood levels dosages in conjunction with appropriate dose-response data. The final dosage regimen will be determined by the attending physician, considering various factors which modify the action of drugs, e.g. the drug's specific activity, the severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding the appropriate dosage levels for the treatment of various diseases and conditions.

A further aspect of the invention involves use of a pyrimidine for the manufacture of a medicament for co-administration with a 4-quinoline-carboxylic acid derivative, and 4-quinoline-carboxylic acid derivative for the manufacture of a medicament for co-administration with a pyrimidine, as well as use of a pyrimidine and a 4-quinoline-carboxylic acid derivative together for the manufacture of a medicament.

The methods of the present invention can be used with other current standard care, such as with other anti-neoplastic agents for cancer treatment, with other anti-inflammatory or immunosuppressive agents such as corticosteroids or non-steroidal anti-inflammatory drugs (NSAIDs) for treatment of inflammatory disorders, and with antibiotics and other immunomodulatory therapy such as cyclosporine A, FK506, corticosteroids, azathioprine, mycophenolic acid, rapamycin, 15-deoxyspergulin, mizoribine, leflunomide, OKT3, anti-IL-2 receptor antibodies, misoprostol, methotrexate, cyclophosphamide and anti-lymphocyte or anti-thymocyte antibodies, for organ transplantation. The methods of the present invention may improve therapeutic effectiveness of these other agents. This may occur through

reducing the amount of agent required to obtain the desired clinical effect, thereby lowering systemic toxicity and/or cost of treatment, and thus allowing wider use of the agents. The present invention may also provide quality of life benefits due to, e.g., decreased duration of therapy, reduced stay in intensive care units or reduced stay overall in the hospital, with the concomitant reduced risk of serious nosocomial (hospital-acquired) infections.

The term "treating" or "treatment" as used herein encompasses both prophylactic and therapeutic use.

"Concurrent administration" or "co-administration" as used herein includes administration of the agents together, or before or after each other. The agents may be administered by different routes. For example, the 4-quinoline-carboxylic acid derivative may be administered intravenously while the pyrimidine is administered intramuscularly, intravenously, subcutaneously or orally. Concurrently, another anti-neoplastic, anti-inflammatory or immunosuppressive agent may also be administered, *e.g.*, intravenously or orally. The agents may be given sequentially in the same intravenous line, after an intermediate flush, or may be given in different intravenous lines. They may be administered simultaneously or sequentially, as long as they are given in a manner sufficient to allow both agents to achieve effective circulating concentrations.

Other aspects and advantages of the present invention will be understood upon consideration of the following illustrative examples. Example 1 addresses the toxic effects of brequinar, with and without uridine, in mice. Example 2 addresses the immunosuppressive effects of brequinar, with and without uridine, in a mouse cardiac allograft transplant model. Example 3 addresses brequinar's effects on tyrosine phosphorylation. Example 4 addresses the immunosuppressive effects of brequinar, with and without uridine, in a mouse lymphoproliferative disorder model. Example 5 addresses the immunosuppressive effects of brequinar, with and without uridine, in a mouse auto-immune disease model.

EXAMPLE 1

Toxic Effects of Brequinar, With and Without Uridine, in Mice

This *in vivo* experiment confirmed the previously reported myelotoxic effects of brequinar that result in anemia and leukopenia. Four to six normal Balb/C mice were treated for six weeks with 10 or 20 mg/kg/day brequinar (Dupont Merck Pharmaceuticals, Wilmington, DE) diluted in saline via intraperitoneal injection to induce anemia. Blood and serum samples were obtained from the mice at weeks 3, 4 and 6. Nucleotide triphosphate and uridine levels in the spleen, bone marrow and serum were quantitated as follows. Spleen or bone marrow (60 mg per sample) were briefly homogenized in 540 μl of 0.4 M tricholoric acid. The nucleotides were extracted by centrifugation and then neutralized with equal volume of 0.4 M tri-n-octylamine in Freon 113 as described [Olempska-Beer and Freese, 1984]. Nucleotides were analyzed in Waters HPLC system with a 616 pump, a 600S gradient controller, a 717 plus autosampler and 996 PDA detector (Milford, MA). The separation was achieved by a linear gradient elution of potassium phosphate buffer, pH 4.5 (10 to 550 mM) on a Whatman strong anion exchange column, partisil 10 SAX (Alltech, Deerfield, IL). The corresponding peaks of four nucleotides were integrated and the concentrations were calculated based on a standard curve of purified nucleotides (Sigma, St. Louis, MO). Serum samples were diluted two-fold in 0.9% NaCl, uridine was extracted by addition of equal volume of 0.8 M tricholoric acid and then neutralized with equal volume of 0.4 M tri-n-octylamine. The level of serum uridine was quantitated in a Waters HPLC system. Tissue uridine was determined with reverse phase HPLC using a Lichrosorb 5-RP-18 column. The column was eluted with 5mM KH_2PO_4, pH 6.8 containing 5% methanol at a flow of 1 ml/min. Uridine was quantified by measurement of absorbance at 280 nm and 254 nm and comparison of the peak height with that of standard solutions.

Treatment with brequinar depressed intracellular pyrimidine nucleotide triphosphate (PyNTP) pools in bone marrow cells, inhibits

hematopoiesis, and induces anemia in Balb/c mice. The hematocrits of these mice, reported as % packed cell volume (PCV) in Figure 1A, were significantly reduced to 44-45% after 4 weeks of treatment with 20-10 mg/kg brequinar, compared to 65% in the untreated group. PyNTP levels were also significantly depressed in bone marrow cells after treatment with brequinar.

When the mice treated with 10 or 20 mg/kg/day brequinar were also co-administered 1000 or 2000 mg/kg/day uridine (Sigma, St. Louis, MO) intraperitoneally for six weeks, the uridine prevented anemia, and the hematocrits remained at levels comparable to untreated controls (61-63%) as shown in Figure 1B. In addition, the co-administration of uridine returned UTP and CTP levels in bone marrow cells to normal as shown in Table 1 below.

Table 1: Effect of uridine co-administration with brequinar on intracellular nucleotides of bone marrow cells *in vivo*. Nucleotide quantitative data is expressed as μg nucleotide/mg protein.

Experimental Group	**UTP**	**CTP**	**ATP**	**GTP**
Bone Marrow	μg/mg protein	μg/mg protein	μg/mg protein	μg/mg protein
Untreated	14.87	4.35	156.06	31.38
10 mg/kg brequinar alone	11.04	3.32	156.22	31.2
2000 mg/kg uridine alone	39.69	10.43	107.64	31.69
10 mg/kg brequinar + 2000 mg/kg uridine	15.24	4.85	150.22	31.04

These observations show that anemia induced by brequinar correlates with depression of pyrimidine nucleotide levels in bone marrow cells, indicating that brequinar's inhibition of bone marrow cell proliferation is mediated through inhibition of DHO-DHase. The co-administration of uridine prevented the induction of anemia by brequinar. Other adverse effects of brequinar that

arise due to inhibition of DHO-DHase, such as bone marrow hypoplasia and lymphocyte depletion, are also expected to be reversed by the co-administration of uridine.

Continuation of brequinar monotherapy for 6 weeks led to a spontaneous reversal in anemia, which was explained by an observed marked enhancement of splenic extramedullary hematopoiesis. In the spleen, the effects of reduced pyrimidine nucleotide synthesis appear to be countered by the salvage of uridine from serum. Levels of serum uridine were observed to be similar in untreated (5.26 ± 2.134 μM uridine; N=4) and brequinar-treated mice (8.55 ± 1.52 μM; N=4). Further *in vitro* studies with PHA-stimulated spleen T cells confirmed that the brequinar-induced depression of intracellular nucleotide pools was prevented, in a dose-dependent manner, by 4-10 μM uridine. Therefore, adequate serum levels of uridine are critical for effective normalization of PyNTP levels *in vivo*; concentrations of ≥ 10 μM may completely reverse the effects of brequinar while concentrations of ≤ 5 μM uridine may be partially effective.

EXAMPLE 2

Brequinar's Activity, With and Without Uridine, in a Mouse Cardiac Allograft Transplant Model

This experiment evaluated whether immunosuppression by brequinar *in vivo* in a mouse cardiac transplant model was mediated by the drug's inhibition of pyrimidine synthesis; if this were so, then co-

administration of uridine at doses that reverse anemia and other toxic effects of brequinar would also reduce its immunosuppressive activity.

The immunosuppressive activity of brequinar was monitored in the Balb/c-into-C3H cardiac allograft model. [Corry et al., *Transplantation*, 16:343 (1973).] Allograft recipients were treated with 2 or 10 mg/kg/day brequinar as monotherapy or in combination with 1000 mg/kg/day uridine, and were monitored for allograft survival. Results are shown below in Table 2. The untreated allografts survived for a mean of 9.2±0.83 days. The 10 mg/kg/day brequinar monotherapy was toxic and the recipients died 3-8 days post-transplant (n=4), while treatment with 2 mg/kg/day brequinar monotherapy extended allograft survival to 47.17 ± 16.4 days. Co-administration of uridine with either 2 or 10 mg/kg/day brequinar prolonged allograft survival to 51.8 ± 16.5 days or 51.0 ± 16.1 days, respectively, and alleviated the fatal toxicity of the high dose brequinar therapy.

Table 2: Uridine does not antagonize the immunosuppressive effect of brequinar

Experimental Group	Allograft Survival	Mean Survival Time ± SD
None	8,9,9,10,10	9.2 ± 0.8
500 mg/kg uridine	8,9,9,10,11	9.4 ± 1.1
10 mg/kg brequinar	3,4,5,8	5.0 ± 2.2
10 mg/kg brequinar + 1000 mg/kg uridine	36,36,61,62,65	52.0 ± 14.7
2 mg/kg brequinar	31,32,34,60,60,66	47.2 ± 16.4
2 mg/kg brequinar + 1000 mg/kg uridine	34,39,48,68,70	51.8 ± 16.5

These results demonstrate that restoring adequate uridine levels reduces the toxicity associated with brequinar administration but has no significant effect on the immunosuppressive activity of brequinar. These data further indicate that brequinar effects immunosuppression by mechanisms independent of inhibition of pyrimidine synthesis.

EXAMPLE 3

Brequinar's Effect on Tyrosine Phosphorylation

This experiment evaluated whether brequinar could inhibit tyrosine phosphorylation in normal murine T cells stimulated with anti-CD3 mAb. T cells isolated from lymph nodes of Balb/c mice were stimulated with 2C11.145 anti-CD3 antibody, 2 μg per sample, 5 x 10^6 cells per sample. The cells were pelleted and directly lysed in NP-40 buffer (50 mM Hepes-HCI, pH 8.0; 150 mM NaC1; 1% Nonidit P-40; 5 mM EDTA; 1 mM sodium vanadate; 5 mM NaF; 1 mM PMSF; 10 μg of aprotinin and leupetinin each). Protein concentration of the postnuclear lysates was measured by using a Bio-Rad protein assay kit (Bio-Rad Lab., Hercules, CA). Thirty μg protein of each sample was separated on a SDS-polyacylamide gel, and then transferred onto nitrocellulose membrane. Protein tyrosine phosphorylation was monitored by Western blot using monoclonal antibody 4G10 (UBI, Placid Lake, NY) and enhanced chemiluminescence (ECL).

The ability of brequinar to inhibit tyrosine kinases *in vitro* was also tested as follows. Immunoprecipitated $p59^{fyn}$ or $p56^{lck}$ from CTLL-4 cells or LSTRA cells (5 x 10^6), respectively, were preincubated with various

concentrations of brequinar in the PTK buffer (50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, and 10 mM $MnCl_2$) on ice for 10 min. Exogenous substrate histone 2B (2 μg) was added and after 20 min, the reaction was initiated by addition of 10 μCi ^{32}[P]-ϒ- ATP. After incubation at 20°C for 10 min, the reaction mixture was subject to electrophoresis on a 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Autophosphorylation of the kinase and phosphorylation of exogenous substrate were analyzed by autoradiography.

Brequinar at a concentration of 25-100 μM was able to significantly inhibit tyrosine phosphorylation induced in murine T cells by cross-linking with anti-CD3 monoclonal antibodies. In addition, the *in vitro* kinase activities of two src-family kinases, *lck* and *fyn* kinases, were inhibited with 50-200 μM brequinar. Although these concentrations are significantly higher than the IC_{50} for inhibition of DHO-DHase (10-50 nM), tissue and serum brequinar concentrations are reported to range from a peak of 200-500 μM to a 24 hour trough level of 20-50 μM in mice treated with a single i.v. dose of 50 mg/kg brequinar. [Shen et al., *Cancer Chemother. Pharmacol.*, 21:183-186 (1988).] Mean levels of brequinar in the sera of rats treated at doses of 3 mg/kg/day are approximately 40 μM, while 24 hour trough levels drop to $\leq$ 10μM. [Eiras-Hreha et al., *Transplant. Proc.*, 1993(suppl.2):32-36 (1993).] The results of this experiment and previous Examples 1-2 show that brequinar has at least two biochemically independent activities that can be defined in vitro; inhibition of DHO-DHase and of tyrosine phosphorylation. Contrary to prevailing assumptions, it appears to be the latter activity that

contributes more to its immunosuppressive activity *in vivo* than the interference with DHO-DHase activity.

EXAMPLE 4

Brequinar's Activity, With and Without Uridine, in a Mouse Lymphoproliferative Model

In MRL-*lpr/lpr* mice, the lymphocytes have a mutated, inactive Fas transmembrane protein and are constitutively proliferative [Zhou et al., *J. Immunol.*, 150:3651-3667 (1993)]. Although these cells do not have the characteristics of malignancy, their Fas protein mutation results in defective apopotosis, persistent cell accumulation and proliferation mainly localized in thymus and lymph nodes. In a preliminary experiment, treatment of MRL-*lpr/lpr* mice (Jackson Laboratories, Bar Harbor, ME) with 10 mg/kg/day brequinar for 4 weeks reduced the weight of lymph nodes and spleen by approximately 80% and 20%, respectively, confirming previous observations that brequinar has anti-proliferative activity against tumor cells and lymphocytes. This dosage of brequinar resulted in moderate toxicity and a body weight loss of about 25%.

Another experiment was conducted to evaluate whether immunosuppression by brequinar *in vivo* in an auto-immune disease and lymphoproliferative model using MRL-*lpr/lpr* mice is mediated by the drug's inhibition of pyrimidine synthesis. MRL (+/+) and MRL-*lpr/lpr* mice were purchased from Jackson Laboratories (Bar Harbor, ME). MRL-*lpr/lpr* mice, 4 in each group at the age of 10 weeks, were treated with 10 mg/kg/day

brequinar alone, 10 mg/kg/day brequinar plus 1000 mg/kg/day uridine, 1000 mg/kg/day uridine alone, for seven weeks by intraperitoneal injection. One group remained untreated, as a control. The 1000 mg/kg/day uridine was given in twice daily doses of 500 mg/kg, since a large dose of uridine would be most likely to prevent brequinar's action if the activity is caused by pyrimidine synthesis inhibition. Four hours after the last treatment with brequinar, the mice were sacrificed. Blood samples were collected, and serum samples were prepared and stored at -80°C. Lymph nodes and thymi were also collected.

Protein tyrosine phosphorylation was determined as follows. About 10 mg of lymph node was directly lysed in NP-40 buffer (50 mM Hepes-HCI, pH 8.0: 150 mM NaC1; 1% Nondit P-40; 5 mM EDTA; 1 mM sodium vanadate; 5 mM NaF; 1 mM PMSF; 10 μg of aprotinin and leupetinin each) , postnuclear lysates were prepared. Protein concentration in cell lysates was measured by using a Bio-Rad protein assay kit (Bio-Rad Lab, Hercules, CA). Thirty μg protein of each sample was separated on a SDS-polyacrylamide gel, and then transferred onto nitrocellular membrane. Protein tyrosine phosphorylation was monitored by using western blot and enhanced chemiluminescence (ECL). The exposed X-Omat films from the *in vitro* tyrosine kinase assays or the phosphotyrosine proteins detected on Western blots were scanned in a LKB densitometer (2202 Ultrascan Laser Densitometer). The peaks corresponding to the bands of interest were integrated to determine the relative amounts of phosphorylation.

The intracellular nucleotide pool was analyzed as follows. Lymph node or thymus (60 mg per sample) were briefly homogenized in 540 μl of 0.4 M trichloric acid, nucleotides were extracted by centrifugation and then neutralized with equal volume of 0.4 M tri-n-octylamine in Freon 113. Nucleotides were analyzed in a Waters HPLC system with a 616 pump, a 600S gradient controller, a 717 plus autosampler and 996 PDA detector (Milford, MA). The separation was achieved by a linear gradient elution of potassium phosphate buffer, pH 4.5 (10 to 500 mM) on a Whatman strong anion exchange column, partisil 10 SAX (alltech, Deerfield, IL). The corresponding peaks of four nucleotides were integrated and the concentrations were calculated based on a standard curve. Serum Sample was diluted two-fold in 0.9% NaCl, uridine was extracted by addition of equal volume of 0.8 M trichloric acid and then neutralized with equal volume of 0.4 M tri-n-octylamine. The level of serum uridine was quantitated in a Waters HPLC system.

The average weight of the lymph nodes and spleen for each treatment group is displayed in Figures 2A and 2B. Treatment with 10 mg/kg/day brequinar monotherapy for 7 weeks reduced the weight of lymph nodes 10-fold, with a slight decrease in spleen weight as well. Uridine treatment alone had no effect on the weight of either organ. Co-administration of 10 mg/kg/day brequinar with 1000 mg/kg/day uridine significantly reduced the size of lymph nodes by 3-fold. These results indicate that the anti-lymphoproliferative activity of brequinar is not solely dependent on its inhibition of pyrimidine synthesis.

Surprisingly, brequinar did not decrease the intracellular pyrimidine nucleotide pools nor the purine nucleotide pools in the lymphocytes prepared from thymus or lymph nodes. This retention of normal levels of pyrimidine nucleotides in the lymphocytes of brequinar-treated MRL-*lpr/lpr* mice is likely due to the rapid salvage of uridine from serum and/or the activation of uridine kinase that converts intracellular uridine to UTP. Quantitation of serum uridine in a HPLC system revealed that brequinar treatment actually increased serum uridine level by 67%, an observation contradictory to previous reports that brequinar decreased the serum uridine level to 37% at 2 hours and 47% at 4 hours after a single administration of brequinar at the dose of 50 mg/kg [Peters et al., *Cancer Res.*, 50:4644-4649 (1990); Peters et al., *Biochem. Pharmacol.*, 1990:135-144 (1990)]. This inconsistency may reflect the difference in the dose of brequinar used in the experiments, or a compensatory elevation in DHO-DHase activity after 4 weeks of treatment, or a decreased demand for nucleotides because of reduced proliferation in the lymphoid compartments.

The hematocrits of the various treatment groups are shown in Figure 3. MRL-*lpr/lpr* mice treated with brequinar alone for 2 weeks became anemic, while co-administration of brequinar with uridine reversed the brequinar-induced anemia observed at 2 weeks. Consistent with the results of Example 1 above, continuous treatment with brequinar alone for 23 days resulted in a spontaneous disappearance of the anemia, although continued brequinar treatment for 53 days resulted in a recurrence of a slight anemia.

Tyrosine phosphorylation was measured in lysates prepared from single cell suspensions or from directly lysed tissue in these animals. Treatment of MRL-*lpr/lpr* mice with brequinar reduced tyrosine phosphorylation of numerous intracellular proteins in lysates of thymus and in the lysates prepared from single cell suspensions of lymph nodes, by more than 70% for several intracellular proteins. Inhibition of protein tyrosine phosphorylation was also observed in the lysates of lymph nodes and in the single cell suspensions of thymus.

These results show that brequinar at a dose effective to control the lymphoproliferative disease of these mice had no effect on the uridine level in serum and the pyrimidine nucleotide levels of lymph organs. These results are in agreement with the previous observations showing that brequinar did not decrease the UTP level in livers and in two tumor cell lines after these tumor-bearing mice were administrated with brequinar at 4 h, through brequinar dramatically inhibited the activity of DHO-DHase in these tissues [Peters et al., *Biochem. Pharmacol.*, 1990:135-144 (1990)], indicating that pyrimidine salvage pathways can provide normal intracellular pyrimidine levels.

Co-administration of uridine slightly reduced but did not eliminate brequinar's control of the expansion of lymph nodes. This result, combined with the observation that brequinar inhibited protein tyrosine phosphorylation in the lymph nodes and thymus, indicates that the anti-lymphoproliferative activity of brequinar is partly mediated by its inhibition of tyrosine phosphorylation.

EXAMPLE 5

Brequinar's Activity, With and Without Uridine, in a Mouse Auto-Immune Disease Model

The MRL-*lpr/lpr* mice of Example 4 also experience an auto-immune disease involving auto-antibody deposition which leads to tissue damage and dysfunction in some organs such as kidney, pancreas and salivary gland. Brequinar's therapeutic effect in auto-immune diseases is partly mediated by its inhibition of auto-antibody production.

Results of monitoring auto-antibody production in the mice of Example 4 are displayed below in Table 3. The serum samples prepared from sacrificed mice were measured for the content of auto-antibody by using an ELISA assay according to Hou et al., *J. Immunol.*, 150:3651-3667 (1993). The results show that brequinar can significantly control anti-DNA IgG and IgM production, and that co-administration with uridine had no effects on the inhibition of auto-antibody production in brequinar-treated animals.

Table 3. Auto-antibody production in MRL-*lpr/lpr* mice treated with various agents

	OD_{405nm} value	
Expt. #1	IgG	IgM
untreated	1.8±0.61	1.81±0.43
10 mg/kg/day brequinar alone	0.025±0.19	0.50±0.26
Expt. #2	IgG	IgM
untreated	1.69±1.08	1.91±0.12
500 mg/kg twice daily uridine alone	1.40±0.77	1.82±0.51
10 mg/kg/day brequinar alone	0.05±0.04	0.43±0.38
10 mg/kg/day brequinar + 500 mg/kg twice daily uridine	0.22±0.21	0.37±0.20

These results show that uridine had no effect on brequinar's inhibition of auto-antibody production, indicating that brequinar's therapeutic effect is not mediated through inhibition of pyrimidine nucleotide synthesis. Thus, inhibition of tyrosine phosphorylation of brequinar also contributes to the control of auto-immune disease in MRL-*lpr/lpr* mice.

Numerous modifications and variations in the practice of the invention are expected to occur to those skilled in the art upon consideration of the foregoing description on the presently preferred embodiments thereof. Consequently the only limitations which should be placed upon the scope of the present invention are those that appear in the appended claims.

WHAT IS CLAIMED ARE:

1. In a method of treating cancer by administration of a 4-quinoline-carboxylic acid derivative, the improvement comprising the co-administration of a pyrimidine.

2. The method of claim 1 wherein the cancer is selected from the group consisting of leukemia, breast cancer, skin cancer, brain cancer, melanoma, colon cancer and lung cancer.

3. The method of claim 1 wherein the 4-quinoline-carboxylic acid derivative is brequinar and the pyrimidine is uridine.

4. The method of claim 1 wherein the 4-quinoline-carboxylic acid derivative is administered at a dose ranging from 1 μg/kg to 100 mg/kg daily, and the pyrimidine is administered at a dose ranging from 1 mg/kg to 5000 mg/kg daily.

5. In a method of treating an inflammatory disorder by administration of a 4-quinoline-carboxylic acid derivative, the improvement comprising the co-administration of a pyrimidine.

6. The method of claim 5 wherein the inflammatory disease is an auto-immune disease.

7. The method of claim 5 wherein the 4-quinoline-carboxylic acid derivative is brequinar and the pyrimidine is uridine.

8. The method of claim 5 wherein the 4-quinoline-carboxylic acid derivative is administered at a dose ranging from 1 μg/kg to 100 mg/kg daily, and the pyrimidine is administered at a dose ranging from 1 mg/kg to 5000 mg/kg daily.

9. In a method of treating a patient who has received an organ transplant by administration of a 4-quinoline-carboxylic acid derivative, the improvement comprising the co-administration of a pyrimidine.

10. The method of claim 9 wherein the organ transplant is an allograft or xenograft.

11. The method of claim 9 wherein the organ transplant is selected from the group consisting of kidney, liver, heart, lung, skin, bone marrow and pancreatic islet cells.

12. The method of claim 9 wherein the 4-quinoline-carboxylic acid derivative is brequinar and the pyrimidine is uridine.

13. The method of claim 9 wherein the 4-quinoline-carboxylic acid derivative is administered at a dose ranging from 1 μg/kg to 100 mg/kg daily, and the pyrimidine is administered at a dose ranging from 1 mg/kg to 5000 mg/kg daily.

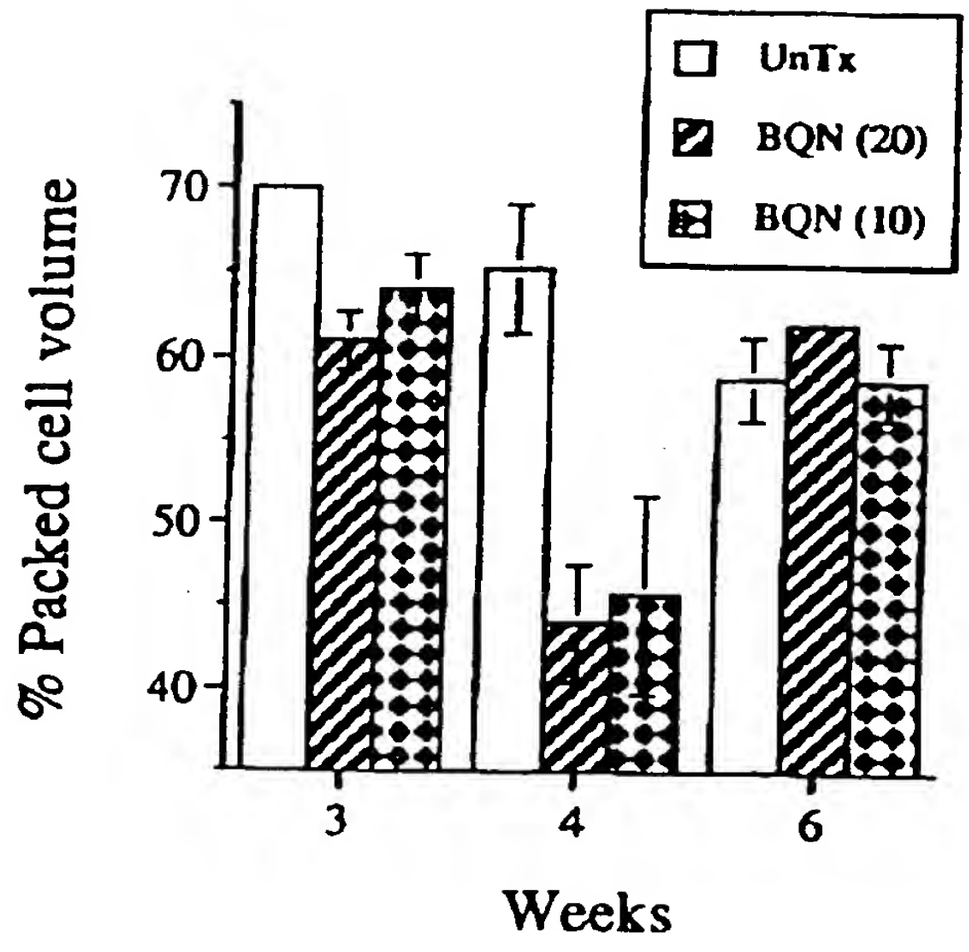

FIG 1a

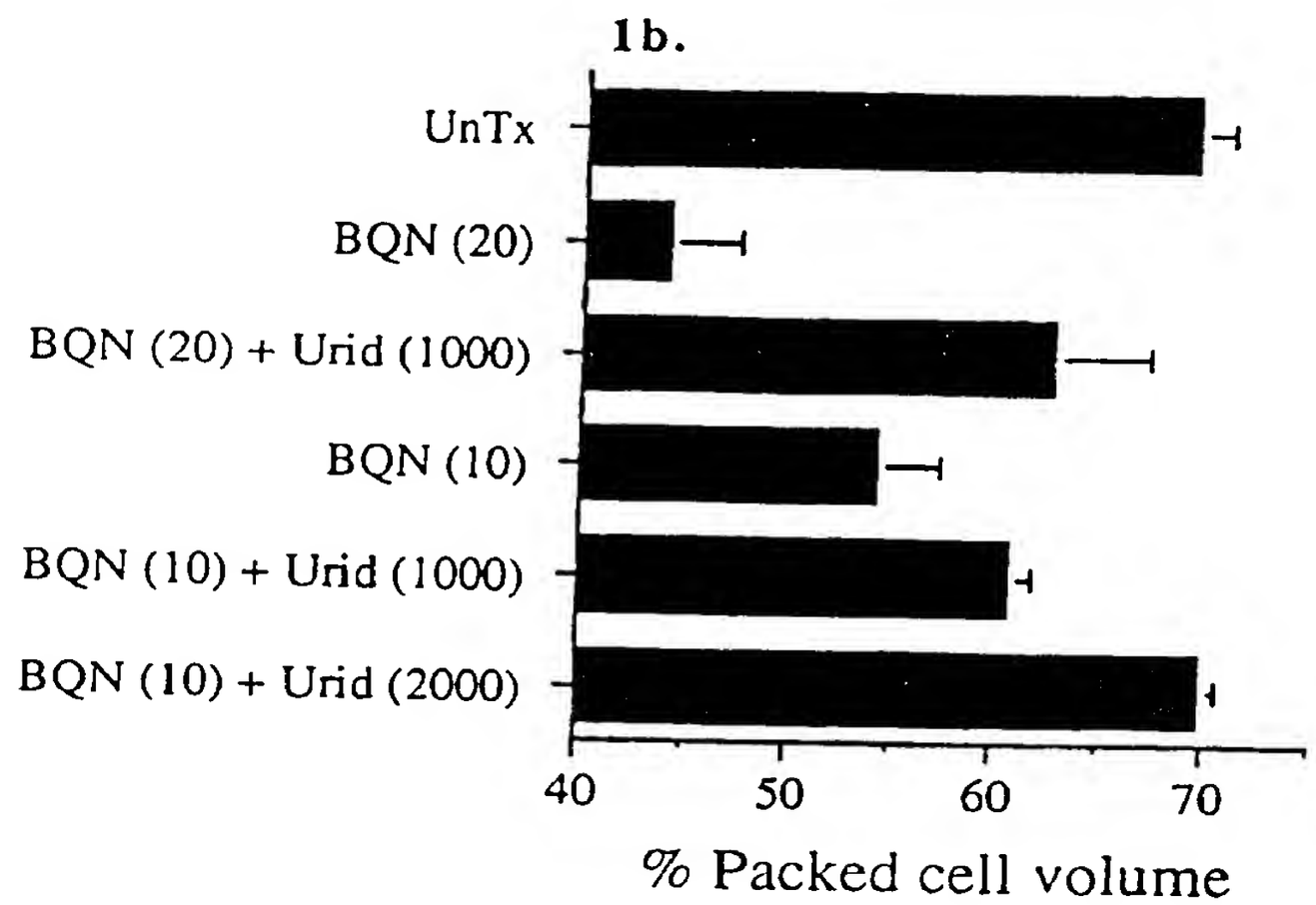
1b.
UnTx
BQN (20)
BQN (20) + Urid (1000)
BQN (10)
BQN (10) + Urid (1000)
BQN (10) + Urid (2000)
40
50
60
70
% Packed cell volume

FIG 1b

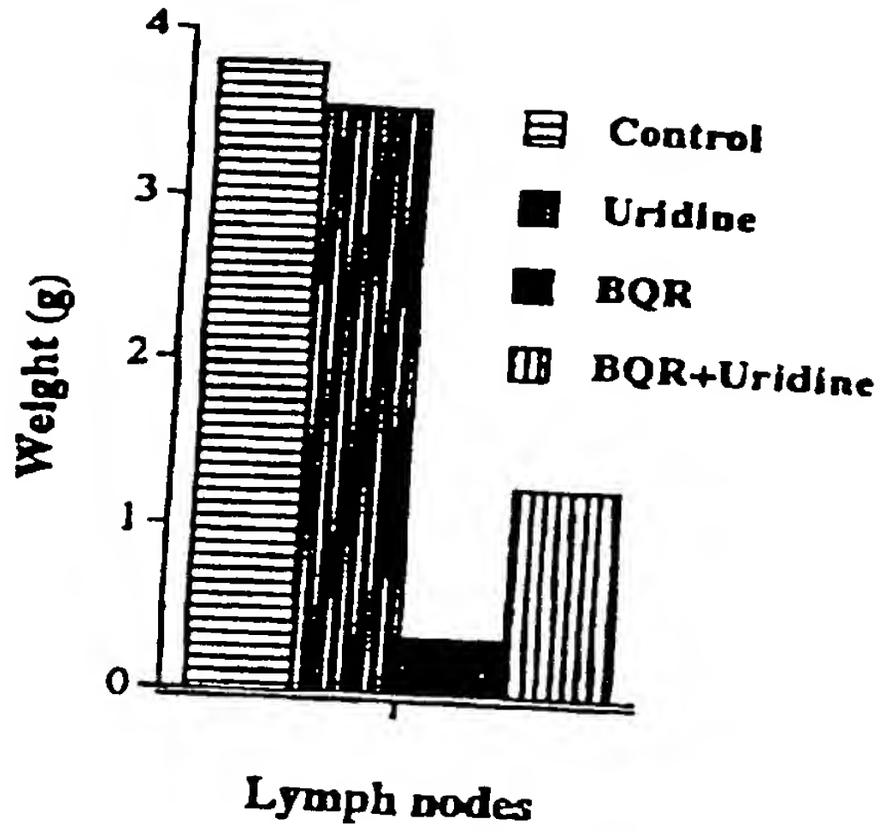

FIG 2a

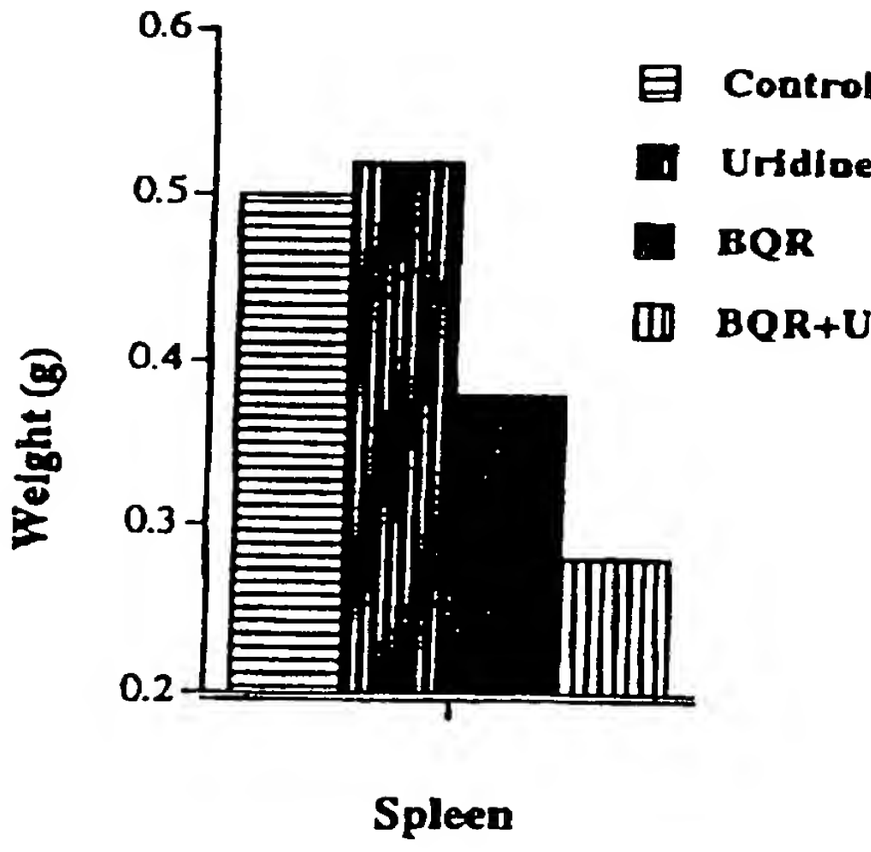
0.6
0.5
0.4
0.3
0.2
Welght (g)
Control
Uridine
BQR
BQR+Uridine
Spleen

FIG 2b

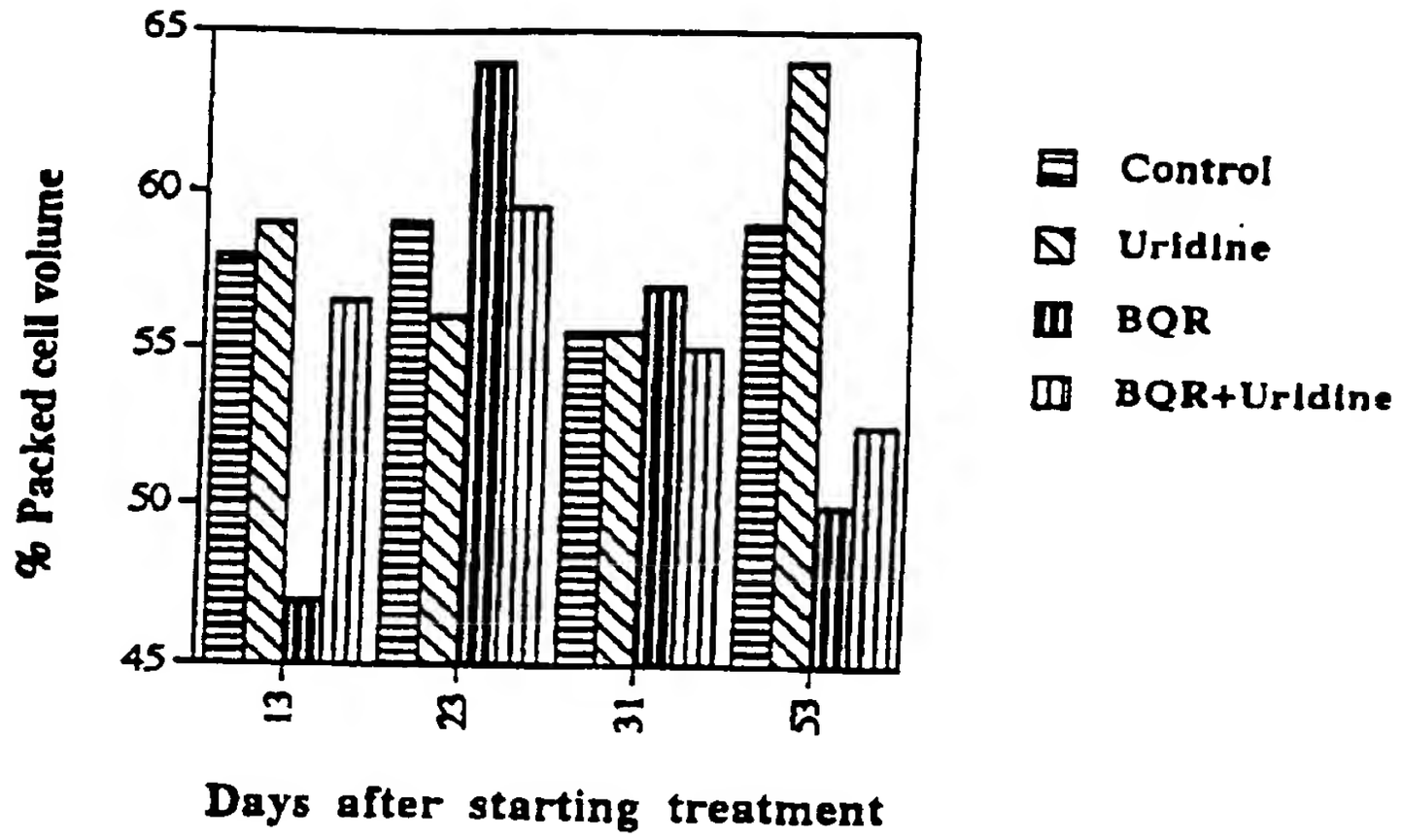
65
60
55
50
45
% Packed cell volume
13
23
31
53
Days after starting treatment
Control
Uridine
BQR
BQR+Uridine

FIG 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17271

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 31/70, 31/47
US CL :514/50, 311
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/50, 311

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database Medline on Dialog, Invest. New Drugs, No. 90-11468, PETERS et al. 'In vitro and in vivo studies on several combinations with Brequinar Sodium' abstract, Vol. 7, No. 4, 1989, page 378. See Abstract.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 10 FEBRUARY 1998	Date of mailing of the international search report 23 FEB 1998
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